EXHIBIT B

Fields
VIROLOGY
Second Edition

Volume 1

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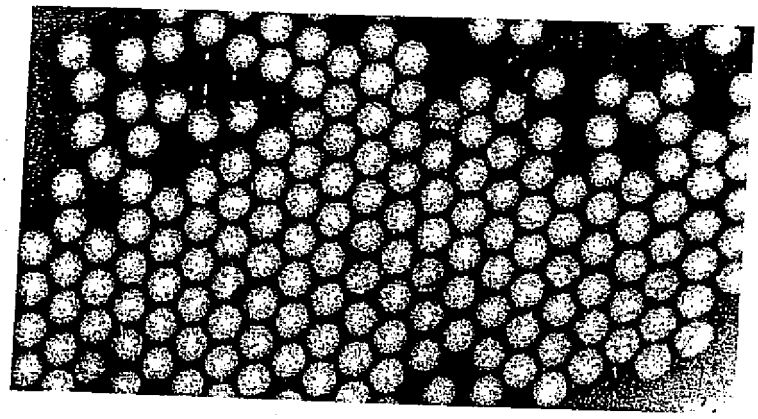

FIG. 1. *Picornaviridae, Enterovirus,* polio 1 virus. ×200,000.

Characteristics: Calicivirus virions are nonenveloped, have icosahedral symmetry, and are 35–40 nm in diameter. Virions have 32 cup-shaped surface depressions in a T = 3 arrangement. The genome consists of one molecule of positive-sense ssRNA, size 8 kb. The viruses have one major polypeptide, MW 65,000, and two minor polypeptides, MW about 19,000. Replication and assembly take place in cytoplasm, and virus is released via cell destruction. The viruses have narrow host ranges.

Human pathogens: *Calicivirus:* possibly Norwalk virus and similar viruses, which are proven causes of gastroenteritis, are caliciviruses. Five distinct caliciviruses have been identified as the cause of gastroenteritis in humans.

Animal pathogens: *Calicivirus:* vesicular exanthema viruses 1–12 of swine, dogs, and mink; San Miguel sea lion viruses 1–8; feline caliciviruses; caliciviruses of calves, swine, dogs, fowl, and chimpanzee.

Togaviridae

Family: *Togaviridae* (the togaviruses) (28) (Fig. 3)
Genus: *Alphavirus* ("group A" arboviruses)
Genus: *Rubivirus* (rubella virus)
Genus: *Pestivirus* (mucosal disease viruses)
Genus: *Arterivirus* (equine arteritis virus)

Characteristics: Togavirus virions consist of a lipid-containing envelope, with fine peplomers, surrounding a nucleocapsid with icosahedral symmetry. Virions are 60–70 nm in diameter. The genome consists of one molecule of positive-sense, infectious, ssRNA, size 12 kb. The viruses have three or four major structural polypeptides, one or two of which are glycosylated. Some of the viruses exhibit pH-dependent hemagglutinating activity. RNA replication involves the synthesis of a complementary negative strand, which serves as a template for genomic RNA synthesis. In alphaviruses, a subgenomic mRNA is synthesized in excess, from which a viral polyprotein is translated and cleaved into the structural proteins. Replication takes place in cytoplasm, and assembly involves budding through host cell membranes. All alphaviruses, but not rubella or the pestiviruses, replicate in arthropods as well as in vertebrates.

Human pathogens: *Alphavirus:* eastern equine encephalitis virus, western equine encephalitis virus,

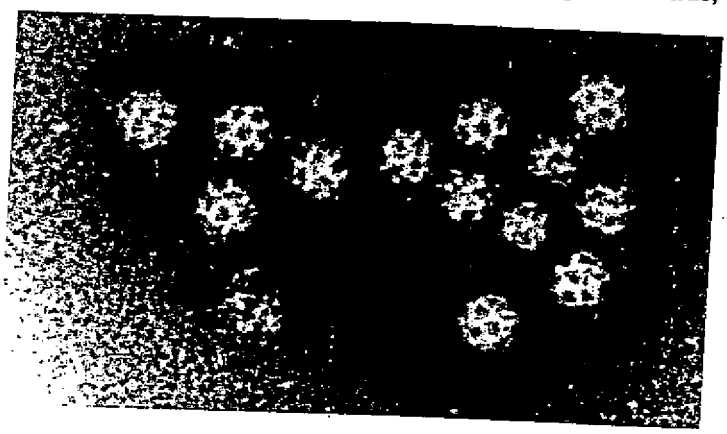

FIG. 2. *Caliciviridae, Calicivirus,* human calicivirus, Sapporo strain. ×177,000.

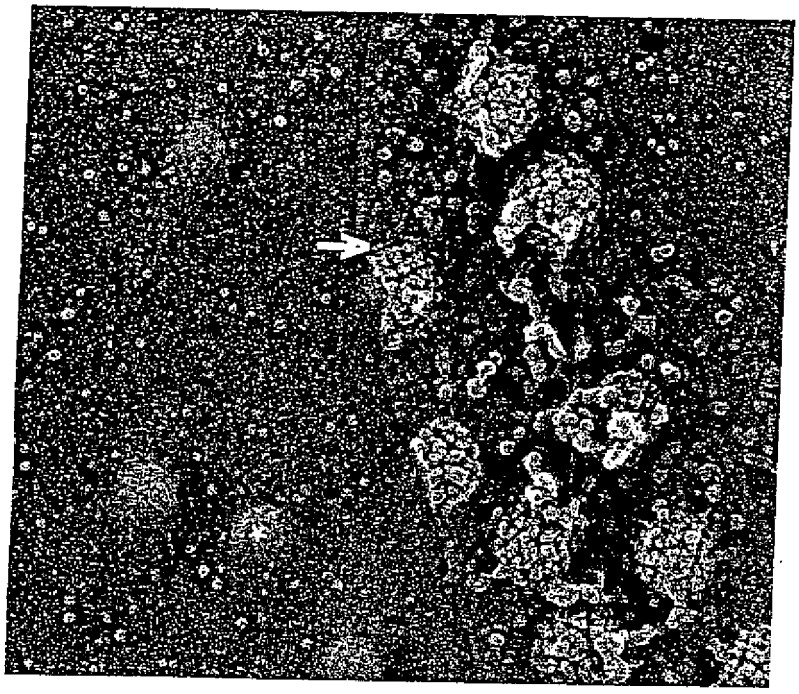

FIG. 5. Budding of Sindbis virions from infected chicken embryo fibroblasts. Cells were quick frozen and freeze fractured. The electron micrograph shows the fracture across the surface membrane of the cell (*white arrow*). On the right is the outer surface of the cell with clusters of virus glycoproteins at sites of budding. On the left is the inner leaflet of the surface lipid bilayer with viral nucleocapsids (*white star*) bound to the inner cytoplasmic side of the membrane. Sample preparation for electron microscopy was carried out by Dr. J. Heuser, Washington University Medical School, St. Louis, MO.

viruses contain DNA genomes or, as with retroviruses, an RNA genome that is replicated through a DNA intermediate. Recent studies with Sindbis virus and two plant RNA viruses (38,77,140,162) have demonstrated that they can serve as expression vectors for the introduction of foreign mRNAs into the cytoplasm of a cell. Two vectors derived from Sindbis virus have been described. The cDNA of a Sindbis DI RNA was engineered to replace the internal viral sequences with foreign sequences including the bacterial chloramphenicol acetyltransferase (CAT) gene. DI RNAs transcribed from this cDNA are replicated and packaged by helper Sindbis virus and become a major viral RNA species in infected cells after passaging (77). They are also translated to produce enzymatically active CAT. In the second vector the structural gene sequences in cDNA clones of the complete Sindbis genome are replaced by the bacterial CAT gene (162). This vector is self-replicating but is only packaged in the presence of helper Sindbis virus. There are several features of Sindbis virus that suggest its usefulness as a vector for the expression of foreign mRNAs. These include not only rapid and efficient expression, but also the ability to grow in a wide variety of cells. Furthermore, the levels of expression can be modulated by the use of temperature-sensitive mutants and adjustments of the temperature at different times after infection. The use of RNA viruses as vectors is in the early stages of development, but the studies with Sindbis virus and the plant viruses show great promise.

Rubiviruses

Rubella virus is the only member of the rubivirus genus (see Chapter 28). This virus is distingushed by its much more limited host range—it does not grow in insect cells—and grows slowly to low titers in cultured cells. In Vero cells, virus release occurs about 48 hr postinfection. Infection is much less cytopathic than that observed with alphaviruses and persistent infections are readily established. Furthermore, assembly of virions may occur predominantly from budding into intracellular organelles such as the ER or Golgi apparatus.

The structure, composition, and overall genome organization of rubella virus is much like that of the alphaviruses; there is a single form of nucleocapsid protein, two envelope glycoproteins, and a single-strand (+) RNA of about 11 kb. The 5′ region of the genome codes for the nonstructural proteins and the 3′ region, expressed as a subgenomic mRNA, encodes the three virus structural proteins (92). On closer examination, however, the rubella genome and consequently the viral proteins proved to be distinct from those of alphaviruses. The cDNA sequence of the rubella subgenomic mRNA shows an unusually high G/C content (142). The derived structural protein sequences have no sequence homology to alphavirus proteins (25, 40,147), although the gene order of 5′ capsid-E2-E1 is identical to that of the alphaviruses. Translation of the subgenomic mRNA produces a polyprotein that

is proteolytically processed by a pathway different from that of the alphaviruses. The capsid is cleaved from the polyprotein by the host signalase and then is released from the membrane by a second proteolytic event. There is no small hydrophobic "linker" protein to serve as a signal sequence for the E1 protein and two forms of about equal size of the E1 glycoprotein appear in virions.

There is a region of 28 nucleotides in the rubella genome that is related in sequence to the junction region of the alphaviruses (40). This region is located 3,346 nucleotides from the 3′ terminus and 96 nucleotides upstream from the initiation codon for the open-reading frame of the structural proteins. Eighteen of the rubella nucleotides are identical to those in the alphavirus junction sequence, suggesting that they comprise the recognition and start site for the rubella subgenomic RNA. The start of the subgenomic RNA, however, as identified by primer extension, is 21 nucleotides downstream of the position expected based on the initiation site of the alphavirus subgenomic RNAs (42).

FLAVIVIRUSES

Flaviviruses have long been known to be the causative agents of a number of human diseases. There are over 60 members that fall into three different groups based on their mode of transmission (see ref. 88 and Chapter 27). About half of them are transmitted by mosquitos, 11 are carried by ticks, and the rest have no known vector. The most prevalent group of mosquito-borne members has been divided into multiple subgroups based on serological cross-reactions.

As visualized in the electron microscope, flaviviruses are 37–50 nm in diameter (91). The chemical composition of a representative flavivirus, St. Louis encephalitis virus, is 6% RNA, 66% protein, 17% lipid, and 9% carbohydrate (143). Flavivirions contain a nucleocapsid core composed of a single-strand, positive-sense RNA genome complexed with a single species of capsid protein. The virion RNA is about 11 kb. The capsid polypeptide is a 13-kd, highly basic polypeptide that is not conserved among flaviviruses. The membrane surrounding the nucleocapsid is composed of a lipid bilayer and two proteins—an M (membrane) protein and the envelope or E protein. The M protein is 7–8 kd and is synthesized as a larger molecular weight glycosylated precursor (prM). The E protein ranges in size from 51 to 60 kd and defines the type specificity of the virus. Many, but not all, of the flavivirus E proteins are glycosylated.

Structure of the Genome

The determination of the complete sequence of the yellow fever genome (104,107) was an important milestone in the history of flavivirus research. These data and the sequence information that is being derived for many of the other flavivirus RNAs including West Nile (21,22,156), Murray Valley (30), St. Louis encephalitis (144), Kunjin (28), Japanese encephalitis (63,135), tick-borne encephalitis (99), and dengue serotypes 1 (85), 2 (33,34,52,57,163), and 4 (82,165) establish that there is a single uninterrupted reading frame covering most of the genome. The genome RNAs lack a poly(A) tail at the 3′ terminus (154) and have a type 1 cap of the form m^7GppAmp at the 5′ terminus (26). For yellow fever RNA the translation initiation codon is located 118 nucleotides from the 5′ terminus, the open-reading frame spans 10,233 nucleotides and is followed by three closely spaced termination codons (104,107). There are 511 untranslated nucleotides at the 3′ terminus. The gene order shown in Fig. 6 is

C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5

This genomic arrangement in which the structural proteins are located 5′ of the nonstructural proteins is reminiscent of the arrangement found in picornaviruses.

There are several regions of the flavivirus genome that are conserved in sequence or structure throughout the family. These include about 90 nucleotides at the 3′ terminus of the genome which can be organized into a stable hairpin loop structure (17,107). Evidence for this structure comes from RNase mapping studies with West Nile virus RNA (17) and from the inability to label flavivirus RNAs at the 3′ end (154). In the proposed structure the 3′-terminal dinucleotide is hydrogen bonded and would be inaccessible to end labeling. There are additional short stretches of conserved and repeated regions in the 3′ noncoding region. The significance of these is unknown, but one stretch of eight contiguous nucleotides in the 3′ untranslated region is complementary to eight contiguous nucleotides at the 5′ terminus of the genome. It has been suggested that these sequences are important in cyclizing the RNA (54,104). Although there is no evidence that flavivirus RNAs can cyclize, alphavirus RNAs (41,69) and bunyaviruses (68) do undergo cyclization and it is thought that this may be an important step in their replication.

There appears to be no extensive sequence conservation at the 5′ termini of flaviviruses. For some of the flaviviruses it has been possible to predict similar hairpin-like secondary structures in this region but there is as yet no independent evidence to support the existence of these structures (15,54).

Viral Replication

The specific steps in flavivirus replication have not yet been described in the detail known for the alphavirus genus of the togaviruses. The initial steps of in-